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# Human Cellular Immune Response to Giardia lamblia

**Summary:** Human peripheral blood mononuclear cells (PBMC) from two individuals experimentally and one naturally infected with *Giardia lamblia* responded strongly (in an *in vitro* lymphocyte proliferation assay) to both heterologous and homologous (parasite origin) *G. lamblia* antigen stimuli. Proliferative responses to specific antigens as determined by T-cell blotting were due to *Giardia* T-cell epitopes mostly present in antigens lower than $M_r$ 85,000 and 31,000 in isolates PM and GS/M-H7, respectively. Additionally, Il-2 production of PBMC respective to T lymphocyte subsets under antigen stimulation were determined in one selected patient. Proliferative and lymphokine responses could be associated with $CD4^+$ PBMC depleted of $CD8^+$ T cells and not with PBMC depleted of $CD4^+$ T cells. These preliminary results suggest the initiation of larger studies addressing questions of cell-mediated immune response and the role of lymphokines in human giardiasis.

**Zusammenfassung:** *Zelluläre Immunreaktion gegen Giardia lamblia beim Menschen.* Periphere Blutmonozyten von zwei Personen mit einer experimentell und einer mit einer natürlich erworbenen *Giardia lamblia*-Infektion zeigten eine ausgeprägte lymphoproliferative Antwort nach *In-vitro*-Stimulation mit Parasitenantigen, das sowohl aus homologen als auch heterologen Parasitenisolaten gewonnen worden war. Eine T-Zell-Blot- Analyse der lymphoproliferativen Immunantwort bezüglich der nach Molekulargewicht aufgetrennten *Giardia*-Antigenkomponenten zeigte, daß das Spektrum der *Giardia*-Antigene mit T-Zell-Epitopen im $M_r$-Bereich von $< 85'000$ für das PM-1-Isolat und $< 31'000$ für das GS/M-H7-Isolat lagen. Bei einem der Patienten wurden Lymphozyten nach antigen-spezifischer *In-vitro*-Proliferation auf ihre Lymphozytensubpopulationen und deren Fähigkeit zur Il-2-Produktion untersucht. Eine lymphoproliferative Antwort, verkoppelt mit einer Il-2-Produktion, war nur bei $CD4^+$ Lymphozyten (nach entsprechender Eliminierung von $CD8^+$ Lymphozyten) und nicht bei $CD8^+$ Lymphozyten (nach entsprechender Eliminierung von $CD4^+$ Lymphozyten) nachweisbar.

## Introduction

*Giardia lamblia* is a protozoan parasite which resides in the small intestines of humans and other mammals. Disease manifestations vary from asymptomatic carriage to severe diarrhea and malabsorption. Although the natural history of *Giardia* infections is not well described, it is known that some persons self-cure, suggesting the development of immunity or resistance, while others develop long-standing chronic infections [1–3]. In humans the importance of the immune system is implied by the increased susceptibility to infection and/or in difficulty with eradiction of infections in patients with hypogammaglobulinemia or X-linked immunoglobulin deficiency [4].

Although both cellular and humoral immune responses have been studied in *Giardia muris* infections in the mouse and suggest the importance of both humoral and cellular mechanisms in the development of protective immunity or resistance to infection [5], there are few studies which suggest the importance of the cellular immune response in humans [1,3,6]. Increased mucosal and intra-epithelial lymphocytes are found in the intestines of humans and *in vitro* studies indicate that phagocytes can ingest opsonized or damaged *G. lamblia*. There is little or no information on the parasite-specific T-cell immune response to *G. lamblia* in humans, despite the idea that the interaction between *G. lamblia* and the cellular immune mechanisms may be important in parasite clearance and in the development of protective immunity as well as contributing to mucosal damage after specific T-cell activation in response to *G. lamblia* infection [3]. Therefore, we have begun to analyze the status of parasite-specific lymphocytes in peripheral blood of patients with naturally or experimentally acquired giardiasis. Although based on a restricted number of investigated patients, our preliminary report suggests that human peripheral T lymphocytes from exposed individuals exhibit a marked lymphoproliferative response to stimulation with a wide range of *G. lamblia* antigenic polypeptides. This cellular immune response includes Il-2 synthesis by $CD4^+$ cells following stimulation with *Giardia* and other control antigens.

## Patients and Methods

*Experimental design*: Cellular immune responses to *G. lamblia* antigens were determined by lymphocyte proliferation assays to both heterologous and homologous parasite isolates in two sets of infected individuals. Later, proliferation to specific antigens was determined by T- cell blotting. Additionally, Il-2 synthesis and the T lymphocyte subsets producing Il-2 under antigen stimulation were determined in one selected patient.

*Patients and control donors*: Peripheral blood mononuclear cells (PBMC) were obtained from the following donors: (i) two

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Table 1: Proliferative response of human PBMC to *Giardia lamblia* antigens.

| Cells from: | Proliferative responses in cpm (stimulation indices, SI) to antigens from *Giardia* isolate | | | | |
|---|---|---|---|---|---|
| | Medium | GS/M-H7 | WEA | PM-1 | PHA |
| Control individuals | | | | | |
| MZ | 102 | 304 (3.0) | 48 (0.5) | 114 (1.1) | 25,955 |
| IC | 349 | 129 (0.4) | 349 (1.0) | 984 (2.8) | 50,369 |
| MM | 1,426 | 4,325 (3.0) | 3,087 (2.1) | 3,779 (2.6) | 31,887 |
| Infected individuals | | | | | |
| PM* | 595 | 29,286 (49.2) | 23,061 (38.8) | 35,748 (60.1) | 31,299 |
| GS** | 665 | 53,705 (80.8) | 28,061 (42.2) | 36,520 (54.9) | 28,560 |
| "Wea"*** | 987 | 14,700 (14.9) | 6,460 (6.5) | 7,420 (7.5) | 31,682 |

* Cells obtained 2 months after natural infection.
** Cells obtained 6 years after natural infection.
*** Cells obtained 2 years after experimental voluntary infection (treated 3 weeks post inoculation).
SI: considered significantly positive when > 3.0.

patients (GS and PM) with coproscopically proven, naturally acquired symptomatic *G. lamblia* infection. Both adult male patients were residents of the Washington D.C. area. Time intervals between infection and blood sampling was six years for GS and two months for PM. GS acquired his infection in Alaska, while PM most likely acquired his infection in the Washington D.C. area (patients PM and GS provided the parasite isolates PM and GS, respectively; the latter was cloned and one clone, GS/M-H7, was subsequently used to infect the normal volunteer "Wea"); (ii) an adult volunteer ("Wea") participated in a previously described study [7]. The volunteer was experimentally inoculated with the *G. lamblia* clone GS/M-H7; he became infected and was treated three weeks post inoculation. The infected volunteer's characteristics have been reported [8]. Blood was obtained two years later. Serum from each of the patients was positive for IgG anti-*G. lamblia* antibodies by ELISA [9] two weeks after infection, but was negative at the time of blood sampling for the present study; (iii) three control persons (MZ, IC, MM) with no history of giardiasis or symptoms suggestive of giardiasis for the preceding six years. They denied current illness, prolonged or undiagnosed diarrhea, and travel to countries with low hygienic standards. MZ (female) and IC (female) were both from Switzerland, MM (male) was from the Washington D.C. area. All control persons were negative for IgG anti-*G. lamblia* antibodies by ELISA.

*Giardia lamblia*: The *G. lamblia* GS/M-H7 clone [10] originated from the patient GS described above, the *G. lamblia* isolate PM-1 and WEA from the patient PM and "Wea", respectively. The *G. lamblia* isolate WEA represented an isolate derived from clone GS/M-H7 after isolation from the experimentally infected volunteer "Wea". The *G. lamblia* clone GS/M- H7 and isolates PM-1 and WEA were cultivated in TYI-S-33 medium with antibiotics as previously reported [11]. Soluble *G. lamblia* trophozoite antigens for use in SDS-PAGE and in stimulation of a lymphoproliferative *in vitro* response were obtained by following exactly the procedure published elsewhere [12].

*Isolation of cells*: PBMC were obtained by sedimentation of heparinized peripheral venous blood on Ficoll-Hypaque (following the manufacturer's instructions, Pharmacia Fine Chem.), washed twice in Hank's balanced salt solution (HBSS), once in tissue culture medium (TCM: RPMI 1640 containing 10% human AB+ serum, 12 mM HEPES buffer, and a supplement of L-glutamine [2mM], penicillin [100 units/ml], streptomycin [100 µg/ml] and fungizone [0.25 µm/ml]). A portion of the cells was diluted 1:1 with freezing medium (50% human AB+ serum and 25% DMSA in TCM as described above) and cryopreserved for supply of feeder cells in some experiments.

*In vitro lymphocyte proliferation assay*: A previously described procedure was employed [12] with the following modifications. Cells were suspended at a concentration of $1.0x10^6$ per ml and tested at $2x10^5$ cells per U-bottomed well. All tests were performed in triplicate. Phytohemagglutinin (PHA) stimulation (1.25 µg PHA per ml) was used as an internal control and PHA was added to the corresponding control wells three days prior to harvesting. Cells were harvested on the sixth day of antigen stimulation and/or *in vitro* cultivation. Results are expressed as mean counts per min (a standard deviation of 20% with respect to the mean cpm was usual) and as stimulation index (SI: mean cpm in experimental wells/mean cpm in medium control wells).

*Il-2-production*: PBMC from one selected, highly responsive *G. lamblia* patient (GS) and from one control person (MZ) were tested for *in vitro* production of Il-2 following stimulation with *G. lamblia* antigen, as well as with the control antigens influenza A virus (FLU), HLA alloantigens [using a pool of 5000 rad irradiated PBMC from three unrelated donors] (ALLO), and PHA. Negative control cultures were unstimulated and contained only media (MED). Cultures used for Il-2 production contained the human anti- Il-2 receptor monoclonal antibody, anti-Tac, to block Il-2 consumption by the activated T cells [13]. The additional control panel of stimuli was selected for study because T helper cell ($T_H$) responses to FLU have been recently shown to be MHC self-restricted and require $CD4^+$ $T_H$ and autologous antigen-presenting cells. In contrast, the $T_H$ responses to ALLO and PHA can utilize both $CD4^+$ and $CD8^+$ $T_H$ [13]. We followed previously established procedures [13] but also included *G. lamblia* antigens which were employed at the concentration mentioned above [12]. After seven days of incubation, culture supernatants were collected and frozen at –20°C. The supernatant Il-2 activity was assessed by the ability to stimulate the proliferation of the Il-2-dependent cell line CTLL. Assay cultures consisted of $8x10^3$ CTLL/well and five successive 2-fold dilutions of supernatant. Results are expressed as mean cpm for test and control wells. Standard deviations were maximally 10% with respect to the mean cpm.


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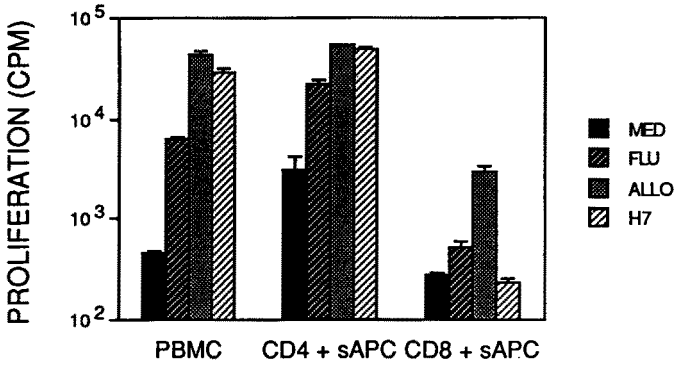


Figure 1: Lymphoproliferative responses of a patient with a past *Giardia lamblia* infection. Unfractionated PBMC (left); PBMC depleted of $CD8^+$ cells and supplemented with (5000 rad irradiated) autologous PBMC as source of accessory cells (middle); and PBMC depleted of $CD4^+$ cells and supplemented with (5000 rad irradiated) autologous PBMC as source of accessory cells (right). The PBMC of patient GS were unstimulated (MED); or were stimulated with influenza A virus (FLU); 5000 rad irradiated allogenic PBMC (ALLO); or *Giardia lamblia* (GS/M-H7 clone) trophozoite antigen. Standard errors of the means are indicated.

*Il-2-determination for $CD4^+$ or $CD8^+$ cells*: PBMC from GS were depleted of $CD4^+$ or $CD8^+$ T cells as described previously [13]. Briefly, $CD4^+$ and $CD8^+$ T cells were negatively selected by a panning technique in which PBMC were incubated with anti-CD4 (Leu 3a) murine MAb (Becton Dickinson, Mountain View, CA). The cells were then washed and incubated for 2 h at room temperature in petri dishes coated with the IgG fraction of goat anti-mouse antibody (Cappel, Cochranville, PA). Non-adherent cells were collected and adjusted to the desired concentrations. These panning techniques permitted 95% depletion as defined by flow cytometry [13]. The subsets were assessed for Il-2-production after antigen stimulation and addition of irradiated autologous PBMC (RC) as an additional source of antigen presenting cells.

*T-cell blotting*: Profiles of PBMC responses to SDS-PAGE resolved, solid-phase (Immobilon®) *G. lamblia* trophozoite antigens were determined by T-cell blotting according to the procedure described previously [12]. PBMC were tested at a concentration of $6x10^5$ cells per microwell. Cells were harvested on the seventh day of antigen stimulation. The results are expressed as the change in counts per min (Δ cpm; mean ct/min in experimental wells – mean ct/min in control wells) or as stimulation index (SI). A maximal standard deviation of 35% with respect to the mean cpm was acceptable [14].

## Results

*In Vitro PBMC Proliferative Response*

PBMC obtained from naturally or experimentally infected patients with giardiasis and control donors were tested for their proliferative response to *G. lamblia* trophozoite antigen preparations derived from the GS/M-H7 clone and from the PM-1 and WEA isolates (Table 1). All patients showed significant proliferation to all three isolates. The two naturally infected patients (GS and PM) exhibited (i) elevated SI with antigen from the homologous infecting parasite isolates/clone and (ii) markedly higher cpm in comparison to the experimentally infected individual ("Wea"). The volunteer "Wea" had higher SI following stimulation with antigens from the

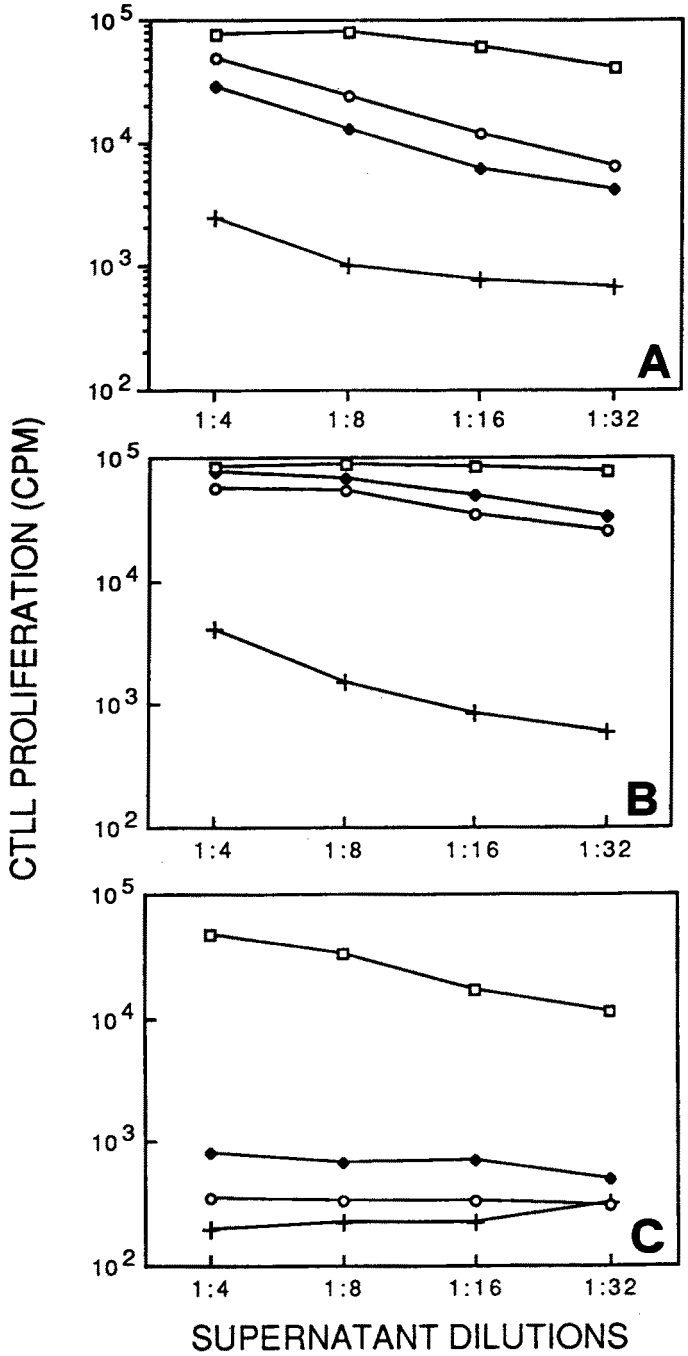


Figure 2: Il-2 titration curves generated by PBMC from patient GS without stimulation (+); or stimulated with FLU (◆), ALLO (□) or *Giardia lamblia* (GS/M-H7 clone) trophozoite antigen (O). The culture supernatants generated in the cultures were diluted through for 2-fold dilutions (1:4 – 1:32) and tested for Il-2 activity on the Il-2 dependent CTLL cell line. Unfractionated PBMC are shown in the upper panel A; PBMC depleted of $CD8^+$ cells in the middle panel B; and PBMC depleted of $CD4^+$ cells in the lower panel C.

## *Giardia lamblia*: Profile of lymphocyte proliferative response in T-cell blotting

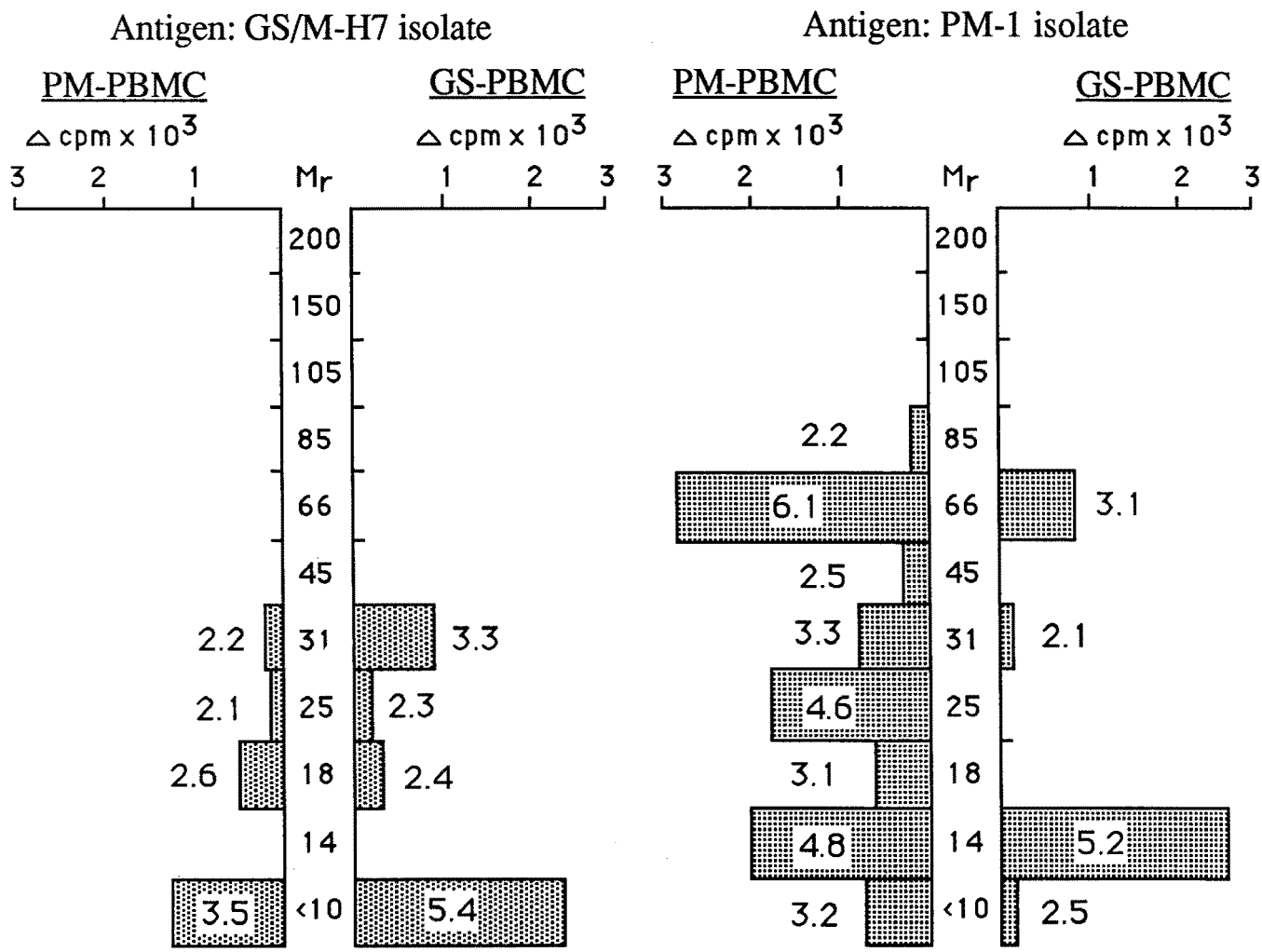


Figure 3: Profile of proliferative responses of PBMC from two (PM and GS) giardiasis patients to stimulation with *Giardia lamblia* antigens derived from the homologous or heterologous infection parasite isolate. The immunoblots consisted of continuous fractions with the $M_r$ indicated on the horizontal axis. The numbers on the side of the columns indicate the SI of the respective fraction. Blank discs without antigen were run in parallel as a negative control (data not shown).

parasite clone (GS/M-H7) which had been used for inoculation in comparison to heterologous isolates.

*Giardia Antigen-Induced Proliferation and Il-2-Production by T-Cell Subsets*

Unfractionated as well as CD8- and CD4-depleted PBMC from patient GS were retested (six and a half years after infection) by proliferation and Il-2 production in response to stimulation with *G. lamblia* (GS/M-H7 clone) trophozoite antigen. In this experiment we also tested the responses of these PBMC to unrelated influenza A virus (FLU) and HLA alloantigens (ALLO), non-*Giardia* antigens to which this patient previously had been shown to be responsive. These two stimuli also served as controls in this experiment, because it had been previously demonstrated that the $T_H$ response to FLU by human PBMC is dependent on $CD4^+$ T cells, whereas $T_H$ activity to ALLO can be mediated by both $CD4^+$ and $CD8^+$ lymphocytes [13].

The proliferative responses are shown in Figure 1. Unfractionated PBMC generated strong proliferative response to all three antigen stimuli. The response to *G. lamblia* (GS/M-H7 clone) trophozoite antigen was stronger than to FLU and almost as strong as to ALLO. PBMC depleted of $CD8^+$ cells ($CD4^+$ sAPC) were responsive to all three stimuli. The unstimulated cultures from this group also exhibited elevated proliferative activity, possibly reflecting an autologous mixed lymphocyte reaction. Nevertheless, the response by unstimulated CD8-depleted PBMC was at least 10-fold below the responses of the stimulated cultures. In contrast to the unfractionated and CD8-depleted groups, PBMC



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depleted of $CD4^+$ cells ($CD8^+$ sAPC) responded to ALLO but not to FLU or *G. lamblia* (GS/M-H7 clone) trophozoite antigen.

The Il-2 titration curves in response to FLU, *G. lamblia* (GS/M-H7 clone) trophozoite antigen and ALLO are plotted in Figure 2. Unfractionated PBMC (upper panel) generated strong responses to all three stimuli. The responses to FLU and *G. lamblia* (GS/M-H7 clone) trophozoite antigen were equivalent and somewhat weaker than the response to ALLO. PBMC depleted of $CD8^+$ cells (middle panel) were strongly responsive to all three stimuli, and the responses to FLU and *G. lamblia* (GS/M-H7 clone) trophozoite antigen were elevated compared to the responses by unfractionated PBMC. In contrast, the Il-2 responses of CD4-depleted PBMC (lower panel) were near background (unstimulated) levels, whereas Il-2 production to ALLO was strong, although somewhat reduced compared to the ALLO response by unfractionated or CD8-depleted PBMC. Thus, the data in Figures 1 and 2 demonstrated that the $T_H$ response to *G. lamblia* (GS/M-H7 clone) trophozoite antigen is mediated by $CD4^+$ T cells but not by $CD8^+$ T cells. It is noteworthy that potent $T_H$ immunity is retained for more than six years after primary infection without any known subsequent exposure to *G. lamblia*.

*T-Cell Blotting*

Unfractionated PBMC were tested by T-cell blotting for their proliferative response to SDS-PAGE resolved solid-phase *G. lamblia* trophozoite antigens from homologous and heterologous infecting parasite isolate/clone. Figure 3 shows the results obtained with PBMC from patients PM and GS, respectively. PBMC from a negative control person (MZ) showed no significant SI>2 over the complete depicted range of the relative molecular mass, $M_r$ (data not shown). The patterns of lymphoproliferative responses of GS and PM PBMC were generally similar with respect to both antigens GS/M-H7 and PM-1, although some differences were noted among the some $M_r$ groupings: For the two patients a group of $M_r$ polypeptides smaller than 85,000 (PM-1-antigen) and 31,000 (GS/M-H7-antigen) stimulated significant proliferative responses. The PM-1-antigen generally appeared to be a stronger stimulus (= higher average counts and wider $M_r$ range) than the GS/M-H7-antigen. The PM-1-antigen also resulted in increased SI with homologous (PM) than with heterologous (GS) PBMC. This difference was not observed with GS/M-H7-antigen.

## Discussion

Specific cellular immune responses to *G. lamblia* and, in particular, to individual resolved parasite antigens have either not been reported, or have only rarely been observed. The present study of lymphocyte proliferative response was performed using peripheral blood leukocytes. Proliferation was stimulated by both soluble antigens from homologous infecting isolates and heterologous *G. lamblia* isolates originating from geographically disparate areas. However, there was a tendency to exhibit higher stimulation indices with the homologous parasite antigen. Cells from patients with naturally acquired infection generally showed much stronger proliferation than those from the experimentally infected person. This appears plausible due to the interruption of infection by chemotherapy three weeks post inoculation, and due to the mild symptoms observed in the voluntarily infected person. In contrast, there were prolonged and marked symptoms for both naturally infected patients. T-cell blotting revealed substantial lymphoproliferative responses over a relative wide $M_r$ (relative molecular mass) range in two naturally infected patients, although this response was restricted to molecules with $M_r < 85{,}000$. This contrasts with a previously published study with similar objectives but employing a mouse model for *G. lamblia* [12], where the $M_r$ range of polypeptides with T-cell epitopes included larger molecules with a dominance at 100–200,000. Remarkable is the lack of a lymphoproliferative response to antigens of the $M_r$ 72,000 area of the GS/M-H7-antigen. This area corresponds to the migration site of the "major surface epitope" of the respective clone [12] and was already characterized by the absence of a lymphoproliferative response in a mouse model [12].

Analysis of parasite-specific lymphoproliferative response and Il-2 production revealed that peripheral cellular and lymphokine reactivity were both dependent on $CD4^+$ T cells (= PBMC depleted of $CD8^+$ T cells).

The role of such cells is unclear, but our results indicate that they maintain a perpetually disseminated parasite-specific memory. This cell-mediated immune response may initially have assisted the induction of (local) humoral immune response. Since there are indications that protective immunity may be acquired [3], and that this and the primary eradiction of *Giardia* from the intestine is dependent on both humoral and cellular responses [15,16], the observed systemic immune response may also reflect an active immunological role and history at the site of infection by direct lymphocyte-parasite interaction. The patients investigated in the present study lacked parasite-specific serum antibodies for two to six years following infection. In contrast, they showed *Giardia*-specific lymphoproliferative and lymphokine responses. Many of the basic questions concerning the role and potential effector mechanisms of immune lymphocytes in clearance of infection and protective immunity remain unanswered. The demonstration of long-lasting cell-mediated immunity (for at least six years) to *Giardia*-antigen raises the possibility of initiating appropriate studies with respect to: (a) local intestinal, humoral and cellular immune responses in human giardiasis patients; (b) participation of cellular immune response in generating protective immunity to reinfection; and (c) consideration of strategies for developing a

prophylactic vaccine against *G. lamblia*. Protective immunity may be highly dependent upon and thus limited by antigenic differences observed within [17] and between various parasite isolates [18]. The strategies for developing a vaccine are suggested by the long-term $T_H$ cell memory observed in the present investigation.

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